Cysteine residues contribute to the dimerization and enzymatic activity of human nuclear dUTP nucleotidohydrolase (nDut)

Shawna M. Rotoli,[1] Julia L. Jones,[2] and Salvatore J. Caradonna[1]*

[1]Department of Molecular Biology, Rowan University, School of Osteopathic Medicine and Graduate School of Biomedical Sciences, New Jersey, 08084, Stratford
[2]Department of Cell Biology, Rowan University, School of Osteopathic Medicine and Graduate School of Biomedical Sciences, Stratford, New Jersey, 08084

Abstract: dUTPase is an enzyme found in all organisms that have thymine as a constituent of DNA. Through evolution, humans have two major isoforms of dUTPase: a mitochondrial (mDut) and a nuclear (nDut) isoform. The nuclear isoform of dUTPase is a 164-amino-acids-long protein containing three cysteine residues. nDut's starting methionine is post-translationally cleaved, leaving four unique amino acids on its amino-terminus including one cysteine residue (C3). These are not present in the mitochondrial isoform (mDut). Using mass spectrometry analyses of recombinant dUTPase constructs, we have discovered an intermolecular disulfide bridge between cysteine-3 of each nDut monomer. We have found that these two residues stabilize a dimer configuration that is unique to the nDut isoform. We have also uncovered an intramolecular disulfide linkage between cysteine residues C78 and C134, stabilizing the monomeric state of the protein. Of note, both disulfide linkages are essential for nDut's enzymatic activity and dimeric formation can be augmented by the addition of the oxidizing agent, hydrogen peroxide to cells. Analyses of endogenous cellular dUTPase proteins confirm these differences between the two isoforms. We observed that mDut appears to be a mixture of monomer, dimer, and trimer conformations, as well as higher-order subunit interactions. In contrast, nDut appeared to exist only in monomeric and dimeric forms. Cysteine-based redox "switches" have recently emerged as a distinct class of post-translational modification. In light of this and our results, we propose that nDut possesses a redox switch whereby cysteine interactions regulate nDut's dUTP-hydrolyzing activity.

*Correspondence to: Salvatore J. Caradonna, Department of Molecular Biology, Rowan University, School of Osteopathic Medicine and Graduate School of Biomedical Sciences, Stratford, NJ 08084, USA. E-mail: caradonn@rowan.edu

Keywords: dUTP nucleotidohydrolase; disulfide bonds; nucleotide metabolism; DNA replication; subunit association; formaldehyde cross-linking; isoforms

Broader word statement

Genomic integrity depends on repairing damaged bases as well as preventing incorporation of inappropriate deoxynucleotides. dUTPase is an enzyme that hydrolyzes dUTP to dUMP, preventing misincorporation of this base. It also provides substrate for *de novo* thymidylate synthesis. Our present work uncovers disulfide linkages in the nuclear dUTPase. These consist of both inter- and intra-cysteine disulfide bonds. Disruption of these linkages leads to dUTPase inactivity. This leads us to propose that this modification may act as a redox switch controlling enzyme activity.

Introduction

Maintaining genomic stability is vital for cell survival. This entails the need for a proper balance of nucleotides for DNA replication and repair. dUTPase is one of the many enzymes which ensures that the integrity of the genome is maintained. It catalyzes the hydrolysis of dUTP to dUMP and pyrophosphate.[1] This reaction is beneficial in two ways: (i) it precludes the buildup of dUTP pools that could otherwise lead to unwanted incorporation of dUMP into DNA and (ii) it provides the substrate for the *de novo* synthesis of TMP.[2,3] dUTPase is an essential gene that is found in all organisms that have thymine as a genomic constituent.

Structurally, dUTPases can be divided into three classes, monomeric, dimeric, and trimeric enzymes.[4] The monomeric forms reside largely within mammalian and avian herpesviruses.[5] The homodimeric enzymes are present in trypanosomes, certain bacteria, and bacteriophages.[6,7] The homotrimeric family is the largest and are found in certain retroviruses and in prokaryotes, as well as eukaryotic cells.[4] In addition to the role of dUTP hydrolysis, dUTPases have been implicated in distinct signaling pathways.[8] Other reports have indicated that the dUTPase enzyme from the Epstein–Barr virus can act as a pathogen-associated molecular pattern protein and can function in immunomodulation.[9,10]

In *Homo sapiens*, the dUTPase gene codes for multiple isoforms. The most prominent forms are a nuclear isoform and a mitochondrial isoform: nuclear (nDut) and mitochondrial (mDut).[11] Due to the alternative use of exons, both proteins contain a unique amino-terminal region while sharing a common carboxyl-terminal domain.[2,11] Multiple crystal structures are available for the human dUTPase enzyme, all of which report a trimeric conformation with three active sites.[3,12–14] These active sites are formed with a combination of the five conserved dUTPase motifs.[3,4,14] Each active site itself contains Motifs 1, 2, and 4 from one monomer, Motif 3 from the second monomer and Motif 5 from the third.[4] The overall tertiary structure of each subunit is composed of antiparallel beta sheets.[4]

Recently, the flexible C-terminal segment of dUTPase has been crystallized, demonstrating its role in the enzymatic activity of the protein.[14] However, a feature common to all current crystal structures is the lack of detail pertaining to the N-terminal arm (Amino acids 1–23 in reference to the nuclear isoform). Also, a secondary finding reported with two of the structures, is the appearance of an asymmetric trimer conformation (1Q5U and 2HQU) where two of the three monomers are more tightly associated.[3,14]

The work presented here largely focuses on nDut, in which the starting methionine is post-translationally cleaved, leaving only four unique amino acids in the amino-terminus when compared with mDut, including one cysteine residue (C3).[15] Results indicate that there is an intermolecular disulfide bridge between the two cysteines at Position 3 of each monomer. These residues stabilize a dimer configuration that is unique to the nDut isoform. A second disulfide bridge has been identified through mass spectrometry analysis between cysteine Residues 78 (C78) and 134 (C134) that stabilize each monomeric subunit.

Finally, we demonstrate that both disulfide bridges are essential for enzymatic activity. In addition, the dimeric state of nDut can be modulated by the addition of the oxidizing agent hydrogen peroxide or the reducing agent beta-mercaptoethanol (BME). Interestingly, recent reports have now identified an additional category of post-translational modifications termed cysteine-based "redox switches."[16] In light of these discoveries, it is possible that nDut may be a part of this enzymatic class. In which, nDut's enzymatic activity is regulated through its redox potential exhibiting "high" enzymatic activity in the oxidized state and "low" activity in the reduced state.

Results

Demonstration of a disulfide linkage in the human nuclear isoform of dUTPase

The serendipitous omission of a reducing agent from an SDS-PAGE/western blot analysis of total cell extracts led us to the finding that the nuclear isoform of dUTPase contains a disulfide linkage. This linkage appears to be between two monomers forming an apparent dimer complex. The intensity of this
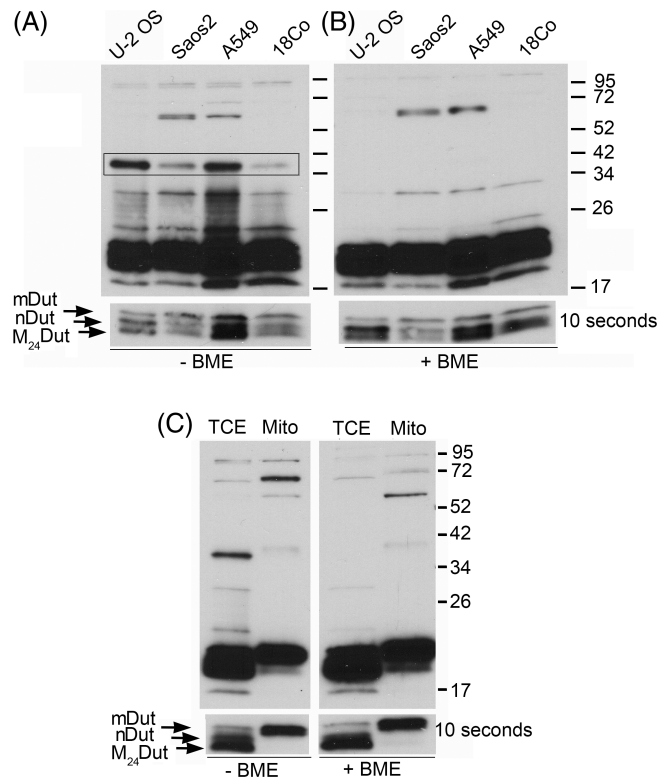
Human ndUTPase Intra- and Intermolecular Disulfide Bonds

Figure 1. Demonstration of intermolecular disulfide bond formation in the nuclear dUTPase protein. (A) A western blot analysis of total cell extracts (TCE) in the absence of the reducing agent, beta-mercaptoethanol (BME) demonstrates a multimeric complex formation in asynchronous populations of U-2 OS, Saos2, A549, and 18CO as indicated by the black box. This complex disappears with the addition of BME in all four cell lines examined. (B) The lower panel in both A and B are exposed to X-ray film for 10 s to demonstrate the monomeric state of the three isoforms of dUTPase. The upper panels are exposed to X-ray film for 1 min. (C) A western blot analysis of TCE and purified mitochondrial extracts (Mito), as indicated, derived from U-2 OS cells ($\pm$) BME shows no multimeric complex formation in the –BME Mito sample. The lower panel is exposed to X-ray film for 10 s while the upper panel is exposed to X-ray film for 2 min. Equivalent amounts of protein were applied to each lane. Blots were probed with a polyclonal specific antibody against the conserved carboxyl-terminal domain of dUTPase. The lower molecular weight band observed in (A) and (B) is likely a truncated form of dUTPase (M24) (Fig. S3).

multimeric complex seems to vary in the cell lines examined. An asynchronous population of the human osteosarcoma cell lines (U-2 OS and Saos2), a human epithelial lung carcinoma cell line (A549), and a human colon fibroblast cell line (18 Co) were extracted in the presence of the alkylating agent, iodoacetamide. This agent was used to negate non-specific disulfide linkages during protein extraction of these cells (Fig. S1). As illustrated in Figure 1(A), a multimeric complex indicative of a dimer is observed (indicated by the black box). This then disappears with the addition of the reducing agent, BME [Fig. 1 (B)] indicating the presence of a disulfide linkage.

There are multiple reported isoforms of human dUTPase. The most prominent forms are a nuclear isoform and a mitochondrial isoform.[11] nDut contains three cysteine residues, Cysteine 3, 78 and 134. C3 is unique to nDut when compared to its mitochondrial counterpart and is located within the flexible n-terminal region of the protein. Whereas C78 and C134 are both located within the protein core approximately 4 Å apart from one another in the tertiary structure.[3] All three cysteine residues are found outside of the five highly conserved dUTPase motifs.[4] The mitochondrial isoform has two cysteines at positions 97 and 153 (based on the mature, processed dUTPase sequence) which are equivalent to the nuclear C78 and C134. A third isoform has been reported to begin at an internal methionine that we have annotated as M24 using the nDut sequence as a reference (NCBI: Accession no. 001025249).

To determine if the mitochondrial isoform contains a disulfide linkage, mitochondria were isolated from U-2 OS and a western blot analysis was performed on proteins separated by SDS-PAGE in the presence and absence of BME. This was then compared to whole cell extracts. As shown in Figure 1(C), it does not appear that mDut forms a multimeric complex through disulfide interactions under these conditions. Considering the fact that all three isoforms contain two of the three cysteine residues but only nDut contains C3, we conclude that C3 is the likely candidate involved in disulfide bond formation leading to the apparent dimer formation. We performed further experimental work to establish the existence of disulfide linkages in the nDut isoform.
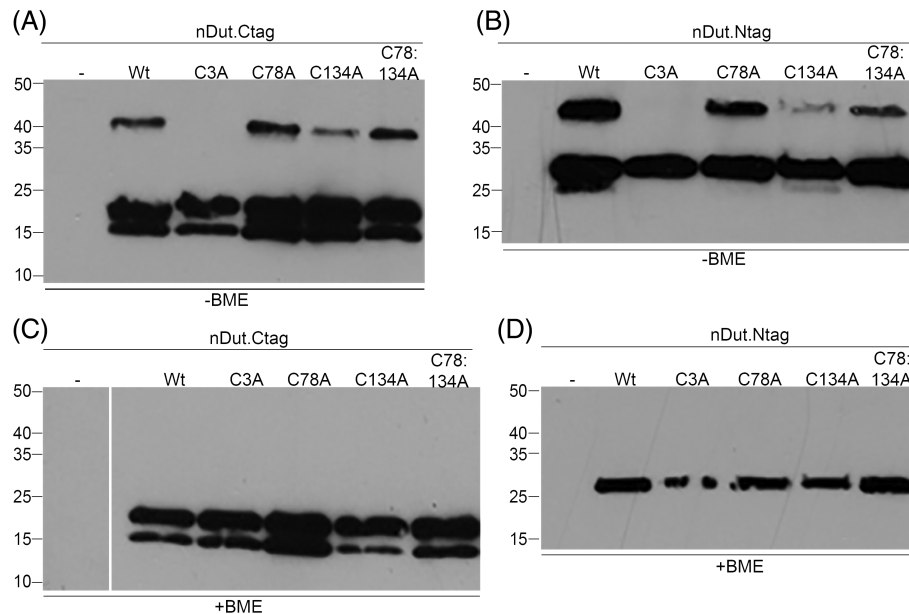
Figure 2. Cysteine 3 is a critical residue in stabilizing the secondary structure of nuclear dUTPase. The expression of nDut.Ctag, nDut.Ntag and cysteine to alanine mutants in U-2 OS cells implicate C3 as a critical residue in stabilizing the higher order structure of n.Dut. Two constructs of each set of proteins contained either a hexahistidine C-terminus tag (A, C) or hexahistidine N-terminus tag (B, D). 3 μg of each plasmid were transiently transfected into U2-OS cells followed by a 24-h incubation. The cells were harvested and 20 μg of total cell extract was applied to a 4–20% tris-glycine SDS-PAGE (±) BME as indicated. Western blot analysis was then preformed using a histidine primary antibody. The predicted molecular weight of nDut.Ntag is 21,188 Da. This includes the existence of a 28 amino acid leader sequence (3458 Da). The nDut.Ctag construct is void of this leader sequence and is predicted to be 18,571 Da. The lower molecular weight band observed in (A) and (C) is likely a truncated form of dUTPase (M24) (Fig. S3).

Identification of the cysteine residues in nuclear dUTPase that form a disulfide linkage

With the novel finding that nDut forms a stable multimeric complex through a disulfide linkage, the next logical step was to elucidate which cysteine residues may be involved. Site-directed mutagenesis of each of the three cysteine residues to alanine was performed. Two sets of three single-mutant constructs (C3A, 78A, and C134A) and one double-mutant construct (C78:134A) were engineered containing either a hexa-histidine C-terminal tag (.Ctag) [Fig. 2(A) and (C)] or a hexahistidine N-terminal tag (.Ntag) [Fig. 2(B) and (D)]. The constructs were transiently transfected into U-2 OS cells for 24 h. Post-transfection the cells were harvested and total cell extracts were prepared. Equivalent amounts of protein were run in an SDS-PAGE gel (±) BME as indicated. Western blot analysis was performed probing for the hexahistidine affinity tag. The results demonstrate that the C3A single-point mutant disrupts the multimeric complex of nDut. As shown in Figure 2, the location of the histidine-tag has no effect on the ability of nDut to form a multimeric complex. The single-mutant C-78A and the double-mutant construct (C78:134A) retain the ability to form a multimeric complex, similar to Wt nDut. The C134A mutant still retains the ability to form the multimeric complex but appears less stable as implicated by the decrease in signal for the complex. This experiment does not negate the notion

that C78 and C134 may have an important role in stabilizing the monomeric state of the protein. When the amino acids are substituted with an alanine it is possible that the folding of the monomeric protein is affected leading to a less stable protein. There is also an observed heterogeneity of the nDut signal in the immunoblots [Fig. 2(A) and (C)]. This may be attributed to an internal start site located at Methionine 24, resulting in a truncated product (M24.Dut; Fig. S3), as discussed earlier. When the tag is located on the N-terminus this heterogeneity is not seen [Fig. 2(B,D)]; again supporting the notion of an internal start site. What can be concluded from this data is that the nDut isoform is able to form a multimeric complex and the cysteine residue at position 3 of the primary sequence is likely responsible for complex formation.

Escherichia coli expressed recombinant nuclear dUTPase verifies the C3–C3 disulfide linkage formation

In order to further understand the structural characteristics of nDut's multimeric nature, Wt and the cysteine to alanine mutants of nDut were expressed in *E. coli* and purified by nickel-chelate affinity chromatography. As shown in Figure 3, protein analysis by SDS-PAGE without BME demonstrates the existence of disulfide linkages in recombinant nDut. The pattern of complex formation of each recombinant
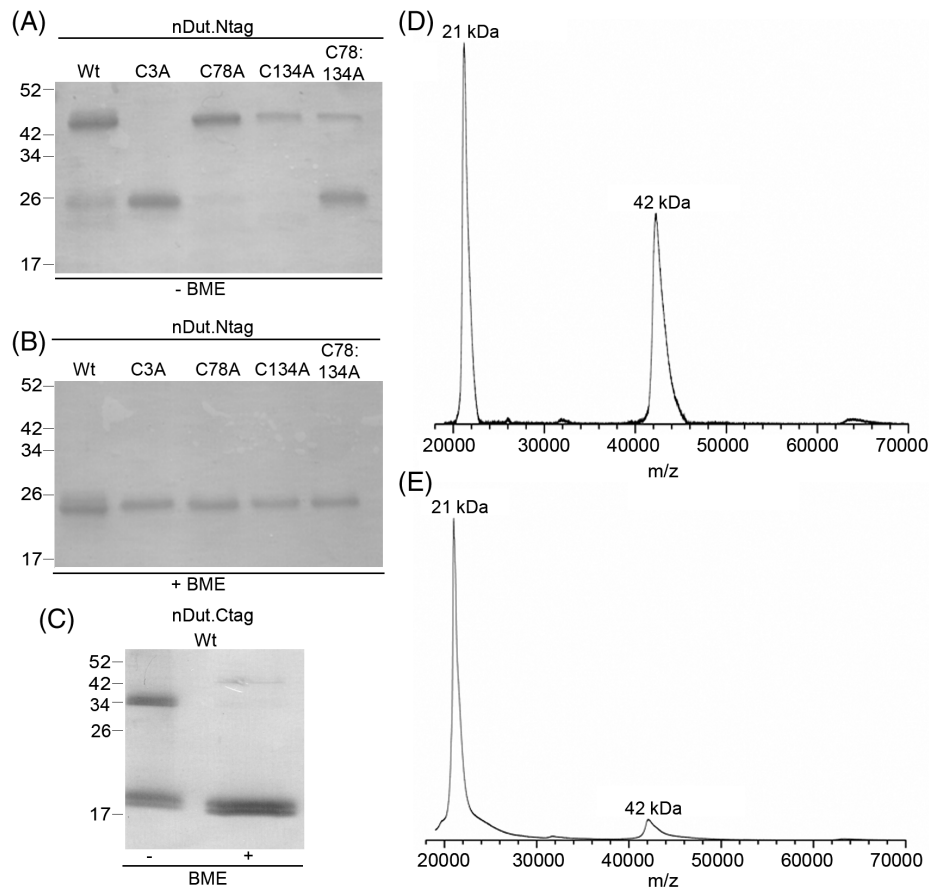
Figure 3. An intermolecular disulfide bridge formation between two cysteine 3 residues is essential for nuclear dUTPase dimer formation. A 12% Tris-Glycine SDS-PAGE coomassie stained gel of recombinant Wt nDut.Ntag and cysteine to alanine mutants in (A) non-reducing (-BME) or (B) reducing conditions (+BME). (C) A 16% Tris-Glycine SDS-PAGE coomassie stained gel of recombinant Wt nDut.Ctag (+/-) BME. Predicted molecular weights and migration behavior on SDS-PAGE is reflective of what is seen in Figure 2. Mass spectrometry was performed with 2 μg of both the full length Wt nDut.ntag (D) and the C3A mutant (E). The peak of interest corresponds to the dimeric (42 KDa) state of the protein which can be visualized in the Wt (D) spectrum. As shown, this peak is significantly diminished in the C3A spectrum.

protein is similar to what is observed in the mammalian expression system, discussed previously. The C3A mutants lack the ability to form any multimeric complexes. While both the single mutants (C78 and C134) are similar to Wt, the double mutant (C78:134A) appears to be more monomeric. This may be due to the additive effects of the mutations possibly disrupting the folding of the monomeric state of the protein, which could then lead to a less stable form, preventing the multimeric complex to form as efficiently.

With the previous knowledge that nDut is phosphorylated on Serine 11,[15,17] we set out to determine if phosphorylation altered the formation of the multimeric complex. A phosphomimetic (S11D) and a phosphorylation blocking (S11A) mutant were expressed in *E. coli* and purified by nickel-chelate affinity chromatography. As shown in Figure S4, protein analysis by SDS-PAGE with and without BME demonstrates no apparent difference between the two mutants when compared with Wt. Leading to the conclusion that phosphorylation does not influence complex formation.

Mass spectrometry supports the finding of a C3–C3 disulfide linkage and uncovers a C78–C134 intramolecular disulfide linkage

To further define the molecular weight of the multimeric complex of nDut that is shown in the previous results, MALDI-TOF mass spectrometry was utilized. Upon analysis of the full-length recombinant nDut.Ntag, a peak was observed that corresponds to the expected molecular mass of the dimeric (42 kDa) form of the protein [Fig. 3(D)] which correlates well to the relative molecular weight observed on the SDS-PAGE gels. To further clarify that this dimeric conformation was due to an intermolecular disulfide linkage between two cysteine 3 residues, a full-length C3A mutant was analyzed [Fig. 3(E)]. The results of this spectrum show what is classically exhibited for a monomeric protein. In this case, a single peak corresponding to 21 kDa [Fig. 3(E)] is observed with a second less intense peak that can be attributed to non-covalent interactions. These results suggest that nDut contains an intermolecular disulfide bridge between two distinct Cysteine

3 residues that covalently stabilize the dimeric state of the protein.

To further confirm the existence of the C3–C3 disulfide bridge MALDI-TOF mass spectrometry was performed using the Wt nDut.Ctag recombinant protein. The Wt nDut.Ctag was alkylated with iodoacetamide, followed by a trypsin-digestion, and then subjected to MALDI-TOF mass spectrometry. The results show an observed peak at 3000.6 Da, a mass which corresponds to the predicted polypeptide fragment (3001.5 Da) of two-linked nDut trypsin digested fragments (PCSEETPAISPSKR) containing the C3 residue (R1-S-S-R1) [Fig. 4(A)], both lacking the first methionine residue. In-source decay (ISD) was used to confirm that the recombinant protein, as reported with the endogenous protein,[15] also lacked the

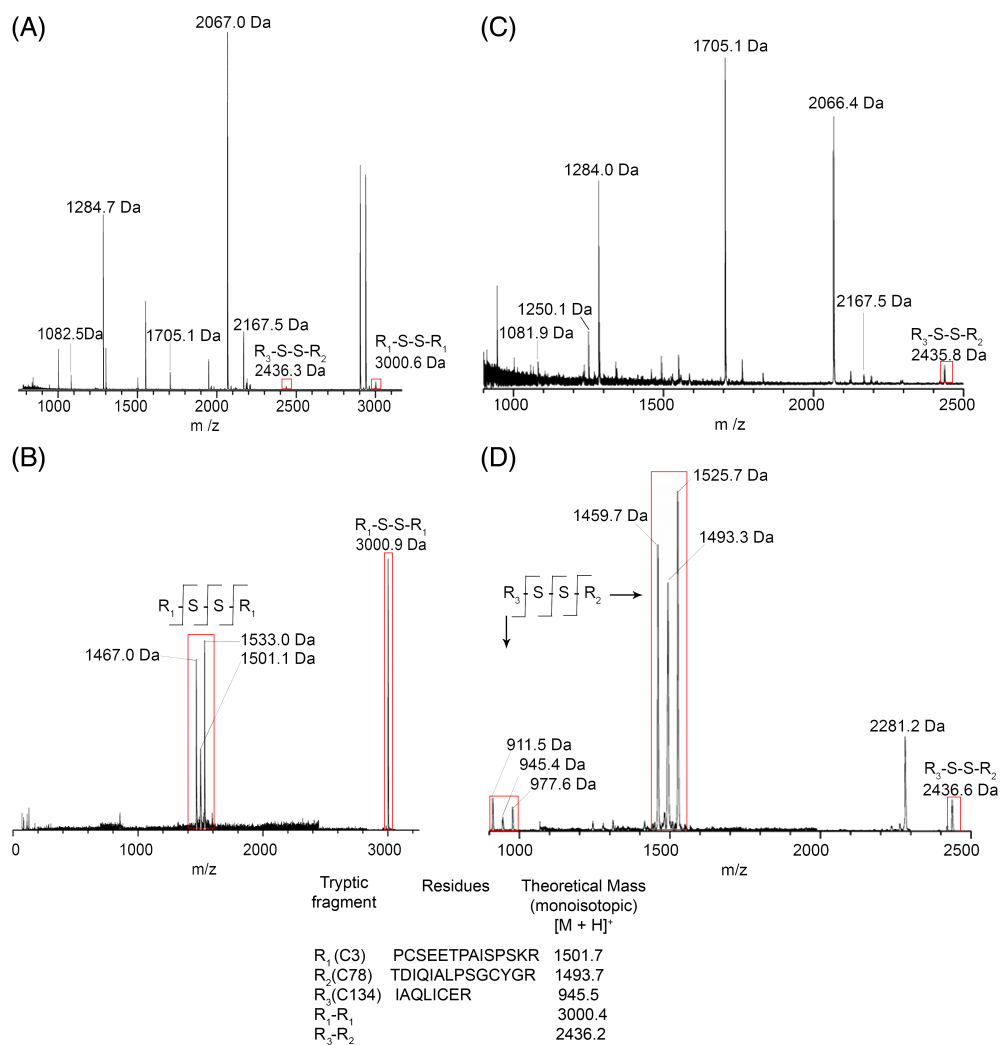| Tryptic fragment	Residues	Theoretical Mass (monoisotopic) [M + H]+
R₁ (C3)	PCSEETPAISPSKR	1501.7
R₂ (C78)	TDIQIALPSGCYGR	1493.7
R₃ (C134)	IAQLICER	945.5
R₁-R₁		3000.4
R₃-R₂		2436.2

Figure 4. Nuclear dUTPase contains both intramolecular and intermolecular disulfide bridges. (A) 20 μg of recombinant n.Dut.Ctag was alkylated with iodoacetamide for 20 min followed by a 3 h trypsin digestion at 37°C. Mass spectrometry analysis was performed on the tryptic fragments. A peak was observed at 3000.6 Da which corresponds to the expected molecular mass of two-linked intermolecular n.Dut tryptic digested fragments containing the cysteine 3 (C3) residue. A second peak was observed at 2436.3 Da which corresponds to the expected molecular mass of two linked tryptic fragments that include cysteine residues 78 and 134. (B) MS/MS analysis verified that the 3000.9 Da peak disassociates into a triple peak at the observed molecular weights of 1467.1, 1501.1, and 1533.0 Da which corresponds to the symmetric and asymmetric cleavage of the disulfide bond that linked the two C3 peptides with a mass separation of approximately 32–34 Da. (C) 20 μg of recombinant n.Dut.ntag was alkylated with iodoacetamide for 20 min followed by an overnight trypsin digestion at 37°C. Mass spectrometry analysis was performed on tryptic fragments. A peak was observed at 2435.8 Da which corresponds to the expected molecular mass of two linked tryptic fragments that include cysteine residues 78 and 134. (D) MS/MS analysis demonstrated the disassociation of that fragment into two distinct species, each containing three peaks which correspond to the symmetric and asymmetric cleavage of the disulfide bond that linked the two peptides. The C78 containing tryptic fragment are clustered at 1459.7, 1493.3, and 1525.7 Da and the C134 containing tryptic fragment are clustered at 911.5, 945.4, and 977.6 Da with a mass separation between each of the peaks of approximately 32–34 Da. The observed peak at 2281.2 Da corresponds to the two linked tryptic fragments observed at 2436.6 Da lacking the weight of one arginine atom (156 Da). In panels A and C, the additional tryptic fragments that are labeled are identified as n.Dut and can been found in Figure S5.
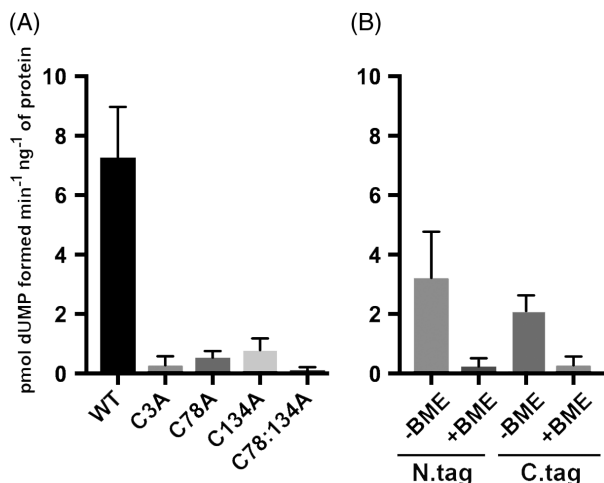
(A)   (B)

Figure 5. Cysteine residues 3, 78, and 134 play an essential role in nuclear dUTPase activity. Each protein was assayed at 37°C for 5 min. The average pmol of dUMP formed per minute per nanogram of protein was then plotted on the y-axis. (A) A substitution of cysteine residues for alanine residues affects the enzymatic activity of nuclear dUTPase, possibly due to disruption of disulfide bonds. Each bar represents an n of 9. (B) Wt n.Dut's enzymatic activity decreases in the presence of the reducing agent BME. All samples for this experiment were allowed to incubate on ice for 15 min with or without 2% BME before performing the assay. Then an additional 2% BME was added directly to the enzyme assay mixture for the indicated samples. Each bar represents an n of 3. All assays were performed at saturating substrate concentrations. Assays were performed in the linear range for both time and protein concentration, based on Wt protein assays. The graphs were generated using GraphPad (La Jolla, CA).

starting methionine residue (data not shown). To verify this intermolecular disulfide bridge, MS/MS analysis of this peak was performed. This analysis shows the dissociation of this fragment into a cluster of three individual peaks, corresponding to the symmetric and asymmetric cleavage of the disulfide bond that linked the peptide with a mass separation of approximately 32–34 Da[18,19] [Fig. 4(B)]. These results are in agreement with previous data showing the existence of an intermolecular disulfide bridge between two monomeric proteins through two C3 residues which stabilize the dimeric state of nDut.

The second observation of an intramolecular disulfide bridge was made while utilizing MALDI-TOF mass spectrometry for Wt nDut.Ctag, as well as Wt nDut.Ntag. Under optimized conditions, a peak was observed corresponding to the mass of two-linked nDut trypsin-digested fragments containing the C78 and the C134 residue (R2-S-S-R3) [Fig. 4(A) and (C)]. What was not observed in either spectra were any peaks that corresponded to a disulfide bond between fragments R1-S-S-R2, R2-S-S-R2, R1-S-S-R3, or R3-S-S-R3. MS/MS analysis of the R2-S-S-R3 peak for Wt nDut.Ntag showed the dissociation of the fragment into two clusters, each with three individual peaks,

corresponding to the symmetric and asymmetric cleavage of the disulfide bond that linked the two peptides[18,19] [Fig. 4(D)]. Together these results indicate the existence of an intramolecular disulfide linkage between cysteine Residues 78 and 134, which likely stabilize the monomeric state of the protein.

Implication of disulfide linkage formation in nuclear dUTPase enzymatic activity

dUTPase plays an essential role in DNA replication and repair. It catalyzes the hydrolysis of dUTP to dUMP + PPi resulting in the decrease of intracellular concentrations of dUTP availability, thus preventing its incorporation into DNA.[1] To investigate the possible roles that the three cysteine residues may have on this enzymatic function, a dUTPase enzymatic assay was used.[1] Recombinant nDut.Ntag hydrolyzed 7.27 pmol of dUTP per minute per ng of protein [Fig. 5 (A)], which is consistent with previously reported enzymatic activity of endogenous dUTPase.[1,20] The enzymatic activity of all mutants decreased when compared with Wt [Fig. 5(A)].

This assay was then repeated using the Wt recombinant protein in the presence of the reducing agent BME [Fig. 5(B)]. As shown in the figure, the presence of a reducing agent drastically reduces enzyme activity, corroborating results presented with the cysteine to alanine mutants. These data show that the cysteine residues are essential for the enzymatic activity, most likely due to the disulfide linkages they form, leading to the stabilization of the overall structural integrity of the protein.

Exploration of higher order subunit interaction of the dUTPase protein

Several crystallographic studies of the human dUTPase protein indicate that this protein forms a trimer.[3,12–14] A number of these studies analyze a truncated recombinant protein that is missing the first 23 amino acids or perform crystallization in the presence of reducing agents.[3,12–14] Under reducing conditions the amino-terminal 23 amino acids would be unstructured and therefore would not appear in the crystal structure. In an effort to determine if a trimeric dUTPase forms in the conditions of our analysis, we performed formaldehyde cross-linking studies with both the mitochondrial and nuclear isoforms of dUTPase purified from U-2 OS and HeLa cells. Figure 6 presents data indicating that the mitochondrial version of dUTPase appears as a mixture of monomer, dimer, and trimer, as well as higher-order subunit interactions. In contrast, nDut produces predominantly monomeric and dimeric proteins as the dominant species. These results allude to the possibility that the two isoforms function as distinct structural entities.
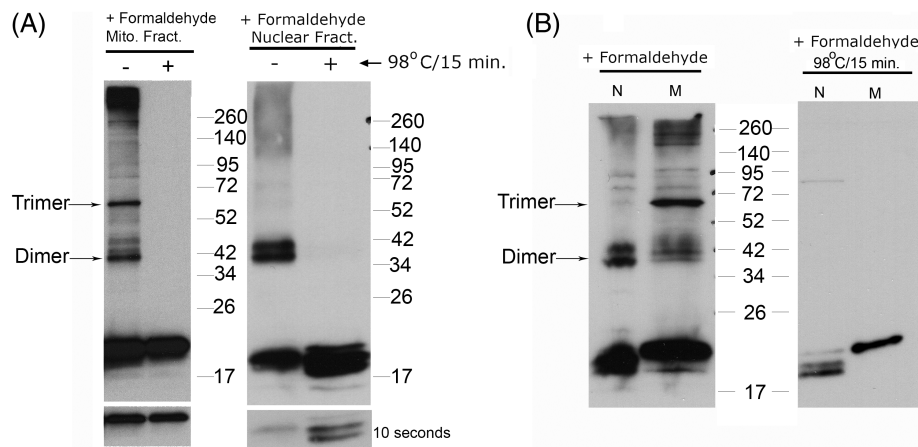
Figure 6. Formaldehyde cross-linking of mitochondrial and nuclear dUTPase demonstrate distinct structural entities for each isoform. (A) HeLa cells and (B) U-2 OS were incubated with formaldehyde as described in materials and methods. Nuclei (N) and mitochondria (M) were isolated then analyzed by western blot using a specific polyclonal antibody against the conserved carboxyl-terminal domain of dUTPase (+ formaldehyde). To reverse the formaldehyde cross-links, extracts derived from both the nuclear and mitochondrial preparations were mixed with SDS-PAGE buffer then heated to 98°C for 15 min in the presence of BME (+ formaldehyde 98°C/15 min). The observed heterogeneity (i.e. doublet bands of nDut) seen with the preparations remain to be explained, but may be due to anomalous migration due to the formaldehyde treatment. Both lower panels in A are exposed to X-ray film for 10 s while B and the upper panels in A are exposed to X-ray film for 2 min.

Consequences of cellular oxidative stress on nDut dimer formation

Thiols in cysteine residues in proteins are among the most oxidant-sensitive targets and can undergo various alterations in response to reactive oxygen species.[21] In an effort to determine if elevated oxidation affects disulfide bond formation in nDut, in particular dimerization, cells in culture were exposed to varying concentrations of hydrogen peroxide for an 18-h period. As seen in Figure 7, there is a four- to five-fold increase in dimer formation as a consequence of hydrogen peroxide exposure. This assay, however, cannot decipher between the oxidized or reduced forms of the monomeric protein. The results outlined in Figure 5 demonstrate that both the C3–C3 intermolecular disulfide bond formation as well as the C78-C134 intramolecular disulfide bond formation are critical for dUTPase activity. Based on these results, it can be inferred that cellular oxidative stress can augment disulfide bond formation in the dUTPase protein and presumably elevate dUTP-hydrolyzing activity.

Discussion

The omission of a reducing agent from an SDS-PAGE/western blot analysis of total cell extract followed by mass spectrometry analysis led us to the finding that nDut contains an intermolecular disulfide bond between C3 residues of two monomers forming a dimer. This dimer configuration is unique to the nDut isoform and is essential for its enzymatic activity. To ensure that the nDut dimer species presented
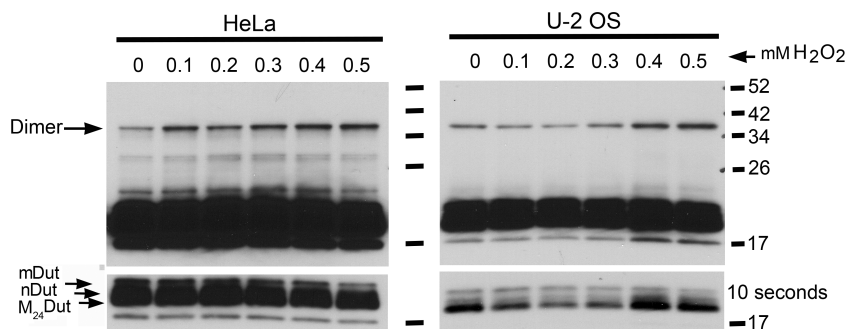

Figure 7. Hydrogen peroxide exposure of cells results in elevated nDut dimer formation. HeLa and U-2 OS cells were exposed to the indicated concentrations of hydrogen peroxide for 18 hours. Cells were harvested followed by protein extraction. 20 μg of protein derived from total cell extract were applied to a 16% SDS-PAGE gel in the absence of the reducing agent, beta-mercaptoethanol (BME), followed by a western blot analysis probed with a specific polyclonal antibody against the conserved carboxyl-terminal domain of dUTPase. The position of dimer is indicated. The upper blots were exposed to X-ray film for 1 min, the lower blots for 10 s. There is a four- to five-fold increase in dimer formation as a function of increasing hydrogen peroxide concentration. ImageJ software was utilized to quantitate band intensity of the dimer.

in our studies is an accurate representation of the endogenous conformational state of the protein and not a consequence of the extraction procedure, iodoacetamide was added directly to the extraction buffer. Our data demonstrate that the C3–C3 disulfide linkage is present post-treatment. It is thought that the overall intracellular environment is in a reducing state,[22] however, it is well documented that microenvironments exist favoring an oxidized state and thus favoring disulfide bond formation intracellularly.[23]

A second disulfide bridge has been identified through mass spectrometry between cysteine residues 78 and 134. These two cysteine residues are located proximal to one another in each monomeric subunit. By disrupting this intramolecular disulfide bridge, the folding of each monomeric protein may be compromised destabilizing the monomeric unit, which then leads to a decrease in enzymatic activity. Each of these disulfide bonds (i.e. C3–C3 and C78–C134) may be regulated differentially by the microenvironment. Further work is needed to elucidate the extent of inter- versus intramolecular disulfide bond formation.

Several reports indicate that nDut is a homotrimeric protein.[3,12–14] Results reported here indicate that only monomeric and dimeric forms are prevalent. It seems feasible that the trimeric state of nDut may be stabilized through both covalent and noncovalent interactions. The dimer first forms through the C3–C3 intermolecular disulfide bridge and then trimer formation occurs through non-covalent interactions between the dimer and a monomer. In an attempt to uncover a trimeric dUTPase, we utilized cross-linking with formaldehyde. Using this reagent, we analyzed both the nuclear and mitochondrial isoforms of dUTPase. mDut appears as a mixture of monomer, dimer, and trimer, as well as higher-order complexes. In contrast, nDut appears to be only in monomeric and dimeric forms. We believe that this difference in structural conformation is due to the unique N-terminal domains of each isoform. The discrepancies between this report and the crystal structures may be due to the stringent conditions needed for crystallization. Future crystallization efforts with the C3–C3 disulfide bond intact may reveal a novel structure for the nDut isoform.

There are slight differences in enzymatic activity between the C-terminal and N-terminal tagged constructs. The decreased enzymatic activity displayed with Wt nDut.Ctag when compared with the N-tagged construct may be attributed to the location of the poly-histidine tag. Domain 5 which is located in the flexible C-terminus tail is thought to be needed to "cap" the active site through multiple interactions with the other domains.[14] It is possible that the addition of the tag may have decreased the flexibility of the C-terminus making these interactions less stable.

Interestingly, recent reports have now identified an additional category of post-translational modifications termed cysteine-based "redox switches".[16] There have been significant strides in understanding this modification. Taking these findings into account, it is conceivable that nDut may now be considered a member of this enzymatic class, where its enzymatic activity is regulated through its redox potential, exhibiting "high" enzymatic activity in the oxidized disulfide state and "low" activity in the reduced sulfhydryl state.

In an effort to expand on this notion, cells were treated with the oxidant, hydrogen peroxide. As seen in Figure 7, there was a four- to five-fold increase in nDut dimer as a function of increasing H_2O_2 concentration. Based on the data shown in Figure 5, dUTP-hydrolyzing activity requires an intact C-3 and presumably an intermolecular disulfide bond formation in nDut. From these observations, it can be inferred that increased cellular oxidative stress leads to increased dimer formation and increased activity of the enzyme.

Lastly, nucleotide metabolism has long been a target for chemotherapeutics, in which drugs such as 5-fluorouracil (5-FU) and fluorodeoxyuridine (FUdR) still continue to see substantial clinical use. They target thymidylate synthases (TS) to induce a severe depletion of TTP pools. However, a major drawback is the drug resistance developed with therapy for drugs of this class. It has been reported that tumor cells are protected from aberrant misincorporation of uracil through the enzymatic action of dUTPase during TS inhibition.[24,25] For this reason, dUTPase has been a target for the development of novel chemotherapeutic reagents.[26] Thus far, the focus of drug development has been on uracil derivatives that bind irreversibly to the active site.[12,13,27,28] When administered they need to be used in combination with multiple chemotherapeutic drugs. With this new structural information, it may now be feasible to target nDut activity through a different mechanism: by disrupting its redox potential by specifically blocking the C3–C3 disulfide linkage rendering the enzyme inactive.

Materials and methods

Cloning

The open reading frame of Wt nuclear dUTPase (Accession no. NM_001948) was cloned into the BamHI-EcoRI restriction sites of the pProEx HTa vector (Invitrogen, Carlsbad, CA) resulting in an N-terminal hexahistidine tag. This includes a 28 amino acid leader sequence (3458 Da) derived from the vector. Site-directed mutagenesis was then used with this construct to create the cysteine to alanine, serine to aspartic acid and serine to alanine mutants using the primers listed in the table. The Wt and cysteine to alanine mutants N-terminal hexahistidine tagged

constructs were sub-cloned into the XbaI-NotI restriction sites of the pCI-neo mammalian expression vector (Promega Corp., Madison, WI).

C-terminal poly-histidine tagged open reading frames of nuclear dUTPase was constructed as follows. Utilizing the primers listed in the table, PCR products of Wt nuclear dUTPase and all mutants were generated containing the C-terminal tag. These constructs were then cloned into the NotI- EcoRI restriction sites of the pCI-neo mammalian expression vector. In contrast to the N-terminal poly-histidine tagged nDuts, these constructs are devoid of the vector leader sequence.

The pProExHTa containing constructs, described above, were utilized to produce bacterially expressed N-terminal tagged protein. To generate C-terminal histidine tagged protein, PCR products were generated from nDut templates using the primers listed in the table. The resulting products were cloned into the NdeI-HindIII restriction sites of the pCW-LIC vector (Addgene plasmid 26098). The resulting constructs encoded the natural methionine start site and a C-terminal histidine tag. DNA sequence analysis was used to verify that all constructs contained the correct open reading frames (University of Michigan DNA sequencing core facility). Primers used for the various cloning procedures are listed in Tables I and S1.

Site-directed mutagenesis

QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) was used for all site-directed mutagenesis reactions. The standard protocol provided by the manufacturer was followed. The C78:134A double mutant was produced by performing a second site-directed reaction on the positive C78A clone with the primers for C134A (Table I). All of the clones were verified via sequencing at the University of Michigan DNA sequencing core facility.

Recombinant protein expression and purification

Wt nuclear dUTPase and mutants were expressed in *E. coli* BL21(DE3)RIPL (New England Biolabs Inc., Beverly, MA) for 1.5 h at 37°C using 1 mM isopropyl beta-D-1-thiogalactopyranoside (IPTG). The cells were harvested at 6000g for 20 min at 4°C. The pellet was washed with 10 mL of PBS then frozen at −80°C overnight. The cells were suspended in Bugbuster reagent (EMD Millipore, Billerica, MA) where the standard company protocol was followed. Nickel column chromatography (Qiagen, Valencia, CA) was used for purification following standard company protocol. Briefly, the column was equilibrated and washed with "Buffer 1" consisting of 50 mM sodium phosphate pH 7.4, 300 mM NaCl, and 10 mM

Table I. *Primers for cloning and mutagenesis*

Primer name	Primer sequence (5′ to 3′)
Wt nuclear dUTPase N-terminal hexahistidine tag (pProEx HTA)	5′-tctagaaccatgtcgtactaccatcaccatca-3′
Wt nuclear dUTPase N-terminal hexahistidine tag Comp (pProEx HTA) Complementary	5′-gcggccgcgctcgtcgacgtaggcctttgaattc-3′
C3A mutant (site-directed mutagenesis)	5′-gggtgtctcttcagaggcgggcatcggatccatg-3′
C3A mutant (site-directed mutagenesis) Complementary	5′-catggatccgatgcccgcctctgaagagacaccc-3′
C78A mutant (site-directed mutagenesis)	5′-gccactcttccataagccccagaagggagcgc-3′
C78A mutant (site-directed mutagenesis) Complementary	5′-gcgctcccttctggggcttatggaagagtggc-3′
C134A mutant (site-directed mutagenesis)	5′-ataaaaaatccgttcggcaatgagctgtgcaattcgatcacctttttg-3′
C134A mutant (site-directed mutagenesis) Complementary	5′-caaaaaaggtgatcgaattgcacagctcattgccgaacggattttttat-3′
Wt nuclear dUTPase, C78A,C134A, and C78;134A C-Terminal hexahistidine tag (pCI-neo)	5′- cgcgaattcatgccctgctctgaagagacac-3′
Wt nuclear dUTPase, C78A,C134A, and C78;134A C-Terminal hexahistidine tag (pCI-neo) Complementary	5′-cgcgcggccgcttaatggtgatggtgatggtgattctttccagtggaaccaaaacc-3′
C3A mutant C-Terminal hexahistidine tag (pCI-neo)	5′-cgcgaattcatgcccgcctctgaagag-3′
C3A mutant C-Terminal hexahistidine tag (pCI-neo) Complementary	5′-cgcgcggccgcttaatggtgatggtgatggtgattctttccagtggaaccaaaacc-3′
Wt nuclear dUTPase C-Terminal hexahistidine tag (pCW-LIC)	5′-gcgcatatgccctgctctgaagagaca3′
Wt nuclear dUTPase C-Terminal hexahistidine tag (pCW-LIC) Complementary	5′-gcgaagctttaatggtgatggtgattctttccagtggaaccaaaacc-3′

Human ndUTPase Intra- and Intermolecular Disulfide Bonds

imidazole. The protein was eluted with "Buffer 2″ containing 50 mM sodium phosphate pH 7.4, 300 mM NaCl, and 150 mM imidazole. The eluted fractions containing the purified protein were pooled and then subjected to overnight dialysis against 50 mM Tris–HCl pH 7.5, 50 mM NaCl, and 10% glycerol. The protein, if needed, was concentrated using Amicon Ultra115 centrifugal filter (UFC901008) using the protocol of the manufacturer.

SDS-PAGE electrophoresis

12%, 16% and 4–20% precast TGX SDS-Page (Bio Rad) or Novex Tris-Glycine gels (ThermoFisher Scientific, Waltham, MA) were used following standard company procedure to separate the proteins in both reducing (1% beta-mercaptoethanol) and non-reducing conditions. Equal amounts of protein from each sample were prepared for SDS-PAGE by the addition of 1 × laemmli SDS-sample buffer (4% SDS, 20% glycerol, 0.004% bromophenol blue, and 0.125 M Tris–HCl, pH 6.8) and heated for 5 min at 85°C unless otherwise specified. For non-reducing conditions, post-formaldehyde cross-linking samples were heated to 37°C for 15 min before loading the gel.

Western blot analysis

Electrophoretically separated protein was transferred to PVDF (Bio-Rad-162-0177). Standard protocols were used to perform the western blot analysis.[29] ECL chemiluminescent western blotting detection system (Amersham Corp., Buckinghamshire, UK) was used to visualize the protein. Blots were exposed to Hyblot CL autoradiography film (Denville Scientific, Holliston, MA) for varying times, indicated in the figures.

Antibodies

dUTPase-specific polyclonal antibody was generated by Covance Research Products (Denver Pennsylvania) based on a recombinant protein provided by our laboratory. DNA sequence encoding the C-terminal portion of dUTPase (DTERGSGGFGSTGKN) was cloned at the C-terminus of glutathione-S-transferase (GST) of the pGEX-3X vector (GE Healthcare Life Sciences, Chicago, IL). The fusion protein was expressed in *E. coli* and purified by glutathione sepharose affinity chromatography using standard protocols of the manufacturer. The purified fusion protein was shipped to Covance for antibody generation in rabbits. Purification of reactive antibody was accomplished by antigen affinity chromatography using a six histidine-tagged dUTPase protein linked to cyanogen bromide-activated sepharose. The purified antibody was stored in PBS at −70°C and routinely used at a dilution of 1:2000 for immunoblot analysis. The antibody recognizes all three isoforms of human dUTPase (Mito. dUTPase, variant 1, Accession no._001025248; Nuclear dUTPase, variant 2, Accession no. _001948; Internal start (Met24) variant 3, Accession no._001025249. Anti-His (HIS.H8) mouse monoclonal antibody was purchased from Thermo-Fisher Scientific (MA1-21315).

Enzyme assays

dUTPase activity was measured using a procedure described by Caradonna and Adamkiewicz,[1] with the alteration of no reducing agent [Dithiothreitol (DTT)] added. For the assays that compared the addition of a reducing agent, each of the protein samples that were tested in reducing conditions had 2% BME added to the protein stock. All samples for this experiment (±BME) were allowed to incubate on ice for 15 min before performing the assay. Then an additional 2% BME was added directly to the enzyme assay mixture for the indicated samples.

Cell culture and related protocols

Saos-2 (ATCC, HTB-85), A549 (ATCC, CCL-185), CCD-18Co (ATCC, CRL-1459), HeLa (ATCC, CCL-2), and U-2 OS (ATCC, HTB-96) cells were grown in Dulbecco's minimum essential medium (GIBCO, Carlsbad, CA), with high glucose containing 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and 1% sodium pyruvate (GIBCO) at 37°C in 5% CO_2. The cells were harvested by scraping and washed twice with PBS. A standard extraction protocol was followed using cell lysis buffer from Cell Signaling Technology with the addition of 5 mM iodoacetamide (ThermoFisher Scientific, Waltham, MA).

Transient transfection: U-2 OS cells were seeded at 1×10^6 in a 60-mm dish 24 h prior to treatment. The cells were then transiently transfected for 24 h with 12 μL TurboFect (Thermo Fisher Inc.), 3 μg plasmid and 600 μL OPTI-MEM (Gibco Life Technology). The mixture was allowed to incubate for 20 min at room temperature before adding it directly to the cells. Post-transfection, the cells were scraped and washed twice with PBS. A standard extraction protocol was followed using cell lysis buffer from Cell Signaling Technology (Boston, MA).

Formaldehyde cross-linking: U-2 OS and HeLa cells were grown to 50–60% confluency in 175 cm^2 flasks. Formaldehyde was added directly to the medium to a final concentration of 1%. The flasks were rocked at room temperature for 15 min and glycine was added to a final concentration of 0.125 M to quench the cross-linking reaction. Figure S2 indicates that this percentage of formaldehyde and the time used produced consistent cross-linked species. Nuclei were prepared from whole cells that had been subjected to formaldehyde. We found that recovery of mitochondria from these cross-linked whole cells was very poor. Therefore, we isolated mitochondria first and then formaldehyde cross-linked as described above.

Cell fractionation for mitochondrial and nuclear fractions: Mitochondria were isolated from U-2 OS and HeLa cells utilizing previously published procedures[11] with the addition of 5 mM iodoacetamide (ThermoFisher Scientific) added directly to the extraction buffer. Homogenization buffer contained 0.25 M sucrose, 1 mM EDTA, 10 mM HEPES, and 0.5% BSA at pH 7.4. Protease inhibitors were added immediately before use. Pellets derived from low-speed centrifugation (600 g/10 min/4°C) were suspended in 0.1% Triton X-100/PBS, incubated on ice for 10 min and then centrifuged. This was repeated once and the pellet was used as the nuclear fraction.

Hydrogen peroxide treatment: U-2 OS and HeLa cells were seeded at 1×10^6 in a 60-mm dish 24 h prior to treatment. Hydrogen peroxide was added directly to the medium to a final concentration of 0.1–0.5 mM as indicated for 18 h at 37°C. Post-treatment, the cells were scraped and washed twice with PBS. A standard extraction protocol was followed using cell lysis buffer from Cell Signaling Technology (Boston, MA).

MALDI-MS

The purified proteins used in mass spectrometry studies were first subjected to dialysis (Pierce Slide-A-Lyzer MINI dialysis unit) for 3 h at 4°C against 100 mM ammonium bicarbonate (Sigma-Aldrich, St. Louis, MO).

MALDI-TOF: Purified nDut.Ntag (2.0 μg) or nDut.Ntag.C3A (2.0μg) was concentrated and desalted using a C4 ZipTip (EMD Millipore, Billerica, MA). The samples were then mixed at a 1:1 ratio with saturated 2,5-dihydroxybenzoic acid matrix (Bruker) in 50% acetonitrile (Sigma-Aldrich) and 0.1% trifluoroacetic acid (TFA) (Sigma-Aldrich) in LL-MS grade water (ThermoFisher Scientific). 1 μl of the mixture was spotted on the MALDI target plate. Positive-ion field reflection mode was used.

MS/MS spectra were measured using Post-Source Decay (PSD). Purified nDut.Ntag (2.0 μg) or nDut. Ctag (2.0 μg) was alkylated for 20 min with 11 mM iodoacetamide (ThermoFisher Scientific) in 100 mM ammonium bicarbonate (Sigma-Aldrich). The protein samples were then treated with 5 μl of 0.1 mg/mL trypsin for 3 h (nDut.Ctag) or overnight (nDut.Ntag) at 37°C. The digest was concentrated and desalted using a C18 ZipTip (EMD Millipore). The samples were then mixed at a 1:1 ratio with 10 μg/μL CHCA matrix (alpha-cyano-4-hydroxycinnamic acid) (Bruker) in 50% acetonitrile (Sigma-Aldrich) and 0.1% TFA (Sigma-Aldrich) in LL-MS grade water (ThermoFisher Scientific). 1 μl of the mixture was spotted on the MALDI target plate. PSD was performed in the positive-ion mode. The range was set for nDut.Ctag at 90 Da and nDut.Ntag at 15 Da.

A Bruker MicroFlex LRF MALDI-TOF instrument was used to collect all data. It has a mass accuracy of <75 ppm in reflector mode and a resolution of >15,000 FWHM (for Somatostatin 28 (m/z 3,147.47)). The laser operates at 337 nm generating a stable ion by a 60 Hz N2-Cartridge-Laser. The ion gate for the precursor ion selection for PSD has a resolution of >100. The instrument was externally mass-calibrated with standards purchased from Bruker. For MALDI-TOF protein standard II was used. For PSD peptide calibration standard II was used. All spectra were analyzed with Flex Analysis software (Bruker).

Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this manuscript.

Contributions
S. R. and S. C. designed the research. Input from J. J. was used for the mass spectrometry studies. S. R. and J. J. performed all the mass spectrometry studies. S. R and S. C. collaborated on all other figures. S. R. and S. C. wrote the paper with input from J. J. All authors analyzed the results and approved the final version of the manuscript.

References
1. Caradonna SJ, Adamkiewicz DM (1984) Purification and properties of the deoxyuridine triphosphate nucleotido-hydrolase enzyme derived from HeLa S3 cells. Comparison to a distinct dUTP nucleotidohydrolase induced in herpes simplex virus-infected HeLa S3 cells. J Biol Chem 259:5459–5464.
2. Caradonna S, Muller-Weeks S (2001) The nature of enzymes involved in uracil-DNA repair: isoform characteristics of proteins responsible for nuclear and mitochondrial genomic integrity. Curr Protein Pept Sci 2: 335–347.
3. Mol CD, Harris JM, McIntosh EM, Tainer JA (1996) Human dUTP pyrophosphatase: uracil recognition by a beta hairpin and active sites formed by three separate subunits. Structure 4:1077–1092.
4. Vertessy BG, Toth J (2009) Keeping uracil out of DNA: physiological role, structure and catalytic mechanism of dUTPases. Acc Chem Res 42:97–106.
5. McGeehan JE, Depledge NW, McGeoch DJ (2001) Evolution of the dUTPase gene of mammalian and avian herpesviruses. Curr Protein Pept Sci 2:325–333.
6. Moroz OV, Harkiolaki M, Galperin MY, Vagin AA, Gonzalez-Pacanowska D, Wilson KS (2004) The crystal structure of a complex of *Campylobacter jejuni* dUTPase with substrate analogue sheds light on the mechanism

and suggests the "basic module" for dimeric d(C/U) TPases. J Mol Biol 342:1583–1597.

7. Hemsworth GR, Moroz OV, Fogg MJ, Scott B, Bosch-Navarrete C, Gonzalez-Pacanowska D, Wilson KS (2011) The crystal structure of the Leishmania major deoxyuridine triphosphate nucleotidohydrolase in complex with nucleotide analogues, dUMP, and deoxyuridine. J Biol Chem 286:16470–16481.

8. Alite C, Humphrey S, Donderis J, Maiques E, Ciges-Tomas JR, Penades JR, Marina A (2017) Dissecting the link between the enzymatic activity and the SaPI inducing capacity of the phage 80alpha dUTPase. Sci Rep 7:11234.

9. Ariza ME, Glaser R, Kaumaya PT, Jones C, Williams MV (2009) The EBV-encoded dUTPase activates NF-kappa B through the TLR2 and MyD88-dependent signaling pathway. J Immunol 182:851–859.

10. Halpin P, Williams MV, Klimas NG, Fletcher MA, Barnes Z, Ariza ME (2017) Myalgic encephalomyelitis/chronic fatigue syndrome and gulf war illness patients exhibit increased humoral responses to the herpesviruses-encoded dUTPase: implications in disease pathophysiology. J Med Virol 89:1636–1645.

11. Ladner RD, Caradonna SJ (1997) The human dUTPase gene encodes both nuclear and mitochondrial isoforms. Differential expression of the isoforms and characterization of a cDNA encoding the mitochondrial species. J Biol Chem 272:19072–19080.

12. Miyahara S, Miyakoshi H, Yokogawa T, Chong KT, Taguchi J, Muto T, Endoh K, Yano W, Wakasa T, Ueno H, Takao Y, Fujioka A, Hashimoto A, Itou K, Yamamura K, Nomura M, Nagasawa H, Shuto S, Fukuoka M (2012) Discovery of a novel class of potent human deoxyuridine triphosphatase inhibitors remarkably enhancing the antitumor activity of thymidylate synthase inhibitors. J Med Chem 55:2970–2980.

13. Miyakoshi H, Miyahara S, Yokogawa T, Chong KT, Taguchi J, Endoh K, Yano W, Wakasa T, Ueno H, Takao Y, Nomura M, Shuto S, Nagasawa H, Fukuoka M (2012) Synthesis and discovery of N-carbonylpyrrolidine- or N-sulfonylpyrrolidine-containing uracil derivatives as potent human deoxyuridine triphosphatase inhibitors. J Med Chem 55:2960–2969.

14. Varga B, Barabas O, Kovari J, Toth J, Hunyadi-Gulyas E, Klement E, Medzihradszky KF, Tolgyesi F, Fidy J, Vertessy BG (2007) Active site closure facilitates juxtaposition of reactant atoms for initiation of catalysis by human dUTPase. FEBS Lett 581:4783–4788.

15. Ladner RD, Carr SA, Huddleston MJ, McNulty DE, Caradonna SJ (1996) Identification of a consensus cyclin-dependent kinase phosphorylation site unique to the nuclear form of human deoxyuridine triphosphate nucleotidohydrolase. J Biol Chem 271:7752–7757.

16. Klomsiri C, Karplus PA, Poole LB (2011) Cysteine-based redox switches in enzymes. Antioxid Redox Signal 14:1065–1077.

17. Lirette R, Caradonna S (1990) Inhibition of phosphorylation of cellular dUTP nucleotidohydrolase as a consequence of herpes simplex virus infection. J Cell Biochem 43:339–353.

18. Tie JK, Mutucumarana VP, Straight DL, Carrick KL, Pope RM, Stafford DW (2003) Determination of disulfide bond assignment of human vitamin K-dependent gamma-glutamyl carboxylase by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. J Biol Chem 278:45468–45475.

19. Jones MD, Patterson SD, Lu HS (1998) Determination of disulfide bonds in highly bridged disulfide-linked peptides by matrix-assisted laser desorption/ionization mass spectrometry with postsource decay. Anal Chem 70:136–143.

20. Ladner RD, McNulty DE, Carr SA, Roberts GD, Caradonna SJ (1996) Characterization of distinct nuclear and mitochondrial forms of human deoxyuridine triphosphate nucleotidohydrolase. J Biol Chem 271:7745–7751.

21. Dalle-Donne I, Rossi R, Giustarini D, Colombo R, Milzani A (2007) S-glutathionylation in protein redox regulation. Free Radic Biol Med 43:883–898.

22. Go YM, Jones DP (2008) Redox compartmentalization in eukaryotic cells. Biochim Biophys Acta 1780:1273–1290.

23. Cumming RC, Andon NL, Haynes PA, Park M, Fischer WH, Schubert D (2004) Protein disulfide bond formation in the cytoplasm during oxidative stress. J Biol Chem 279:21749–21758.

24. Ladner RD, Lynch FJ, Groshen S, Xiong YP, Sherrod A, Caradonna SJ, Stoehlmacher J, Lenz H-J (2000) dUTP nucleotidohydrolase isoform expression in normal and neoplastic tissues: association with survival and response to 5-fluorouracil in colorectal cancer. Cancer Res 60:3493–3503.

25. Webley SD, Hardcastle A, Ladner RD, Jackman AL, Aherne GW (2000) Deoxyuridine triphosphatase (dUT-Pase) expression and sensitivity to the thymidylate synthase (TS) inhibitor ZD9331. Br J Cancer 83:792–799.

26. McIntosh EM, Haynes RH (1997) dUTP pyrophosphatase as a potential target for chemotherapeutic drug development. Acta Biochim Pol 44:159–171.

27. Miyakoshi H, Miyahara S, Yokogawa T, Endoh K, Muto T, Yano W, Wakasa T, Ueno H, Chong KT, Taguchi J, Nomura M, Takao Y, Fujioka A, Hashimoto A, Itou K, Yamamura K, Shuto S, Nagasawa H, Fukuoka M (2012) 1,2,3-Triazole-containing uracil derivatives with excellent pharmacokinetics as a novel class of potent human deoxyuridine triphosphatase inhibitors. J Med Chem 55:6427–6437.

28. Miyahara S, Miyakoshi H, Yokogawa T, Chong KT, Taguchi J, Muto T, Endoh K, Yano W, Wakasa T, Ueno H, Takao Y, Fujioka A, Hashimoto A, Itou K, Yamamura K, Nomure M, Nagasawa H, Shuto S, Fukuoka M (2012) Discovery of highly potent human deoxyuridine triphosphatase inhibitors based on the conformation restriction strategy. J Med Chem 55:5483–5496.

29. Fischer JA, Caradonna S (2011) Analysis of nuclear uracil-DNA glycosylase (nUDG) turnover during the cell cycle. Methods Mol Biol 761:137–149.